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Thermal Resistance of Spores from Five Type E *Clostridium botulinum* Strains in Eastern Oyster Homogenates

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ABSTRACT

The thermal resistance of spores from five type E *Clostridium botulinum* strains, Alaska, Minnesota, G21-5, 25V-1 and 25V-2, in oyster homogenates was determined at 73.9, 76.7, 79.4, and 82.2°C. Thermal death times (TDT) were determined in TDT tubes containing 1-g sample and heated using a constant temperature water bath for different time intervals. D values ranged from 0.07 to 0.43 min at 82.2°C (180°F) and from 2.00 to 8.96 min at 73.9°C (165°F). One strain (Minnesota) isolated from a botulism outbreak in Minnesota was the most heat resistant while strains isolated from crabs (G21-5, 25V-1, and 25V-2) were least resistant. The z values were 4.2 to 5.4°C for strain (Alaska) associated with an outbreak and 6.0 to 7.1°C for the other four strains. Results indicate that these organisms are less heat resistant in oyster homogenate than other seafood products. However, current oyster pasteurization methods are not sufficient to guarantee safety from type E. *C. botulinum* spores, and further studies are needed to assure product safety.

Most oysters in the United States are marketed as freshly shucked. Declining oyster resources in the Chesapeake Bay area during the past half century threaten the oyster processing industry. The situation is further exacerbated by the short shelf life of fresh oysters. Alternative production or processing approaches could promote the development of this industry. By combining chlorination, packaging in flexible pouches, pasteurization, and cold storage, a process for oysters is achieved which extends their shelf life from 2 weeks to 3 months (*6*). Although no case of botulism from Chesapeake Bay oysters has been reported, there is no assurance that pasteurized oysters are clostridia free (*24*). Therefore, a major concern regarding safety of pasteurized oysters is outgrowth and toxin production by *C. botulinum* type E.

C. botulinum type E was first reported by Gunnison et al. (*15*) and isolated by Kushnir et al. (*19*) from the intestines and muscles of sturgeon. Since then, numerous outbreaks of type E botulism in seafood products including fish and shellfish have been reported worldwide (*4,5,17*). The organism has been detected in seafood, other marine animals, sediment and water (*2,9,12,16,23,26,30*). This organism is relatively heat sensitive and capable of germination, multiplication, and production of toxin at temperatures as low as 3.3°C (*8*). Heat resistance of type E *C. botulinum* has been studied in crabmeat, smoked fish, and ground whitefish chubs (*7,10,17,18,22*). However, little is known about the heat resistance of this organism in oyster meat except for our unpublished report (*20*) and a recent paper regarding strain Beluga (*3*).

This study was conducted to determine the thermal death time (TDT) and z value of five strains of type E *C. botulinum* spores including isolates from the Chesapeake Bay and botulism outbreaks in the northern United States.

MATERIALS AND METHODS

Strains and spore suspension

Five test strains, Alaska, Minnesota, G21-5, 25V-1, and 25V-2, were provided by Donald A. Kautter, Food and Drug Administration. Strains Alaska and Minnesota were originally isolated from seafood botulism outbreaks in Alaska and Minnesota (*7,11*). G21-5, 25V-1, and 25V-2 strains were isolated from gills or viscera of crabs taken from the James River estuary in the lower Chesapeake Bay (*22*).

For preparation of spores, 0.2 ml of each culture was inoculated into a 10-ml screw-capped tube containing 8 ml of freshly prepared trypticase-peptone-glucose-yeast extract (TPGY; trypticase, BBL, Cockeysville, MD; all others, Difco products, Detroit, MI) broth and incubated at 28°C for 2 d. The contents of tube culture were transferred into 80 ml of TPGY broth in a 100 ml Wheaton bottle and incubated at 28°C for 1 d. The 80-ml culture was transferred into 800 ml of TPGY in a 1-L Wheaton bottle and incubated at 28°C for 1 week. Spores were harvested by centrifugation at 5,000 x 9 for 10 min and washed three times in distilled water. Washed spores were resuspended in distilled water to a final concentration of 10^5 to 10^7/ml determined by the most probable number (MPN) method and kept at 2°C for use. Spores were examined under microscope for their purity and enumerated by the 3-tube MPN technique with serial 10-fold dilutions in TPGY broth. Vegetative cells were not considered for study because they were unlikely to survive under these conditions of spore preparation (*13,20,22,29*).

Oyster homogenate

Live Eastern oysters (*Crassostrea virginica*) harvested from the Choptank River, 4 miles from Cambridge, MD, were washed,

shucked within 3 h and drained. Freshly shucked oyster meat was homogenized in a Waring blender for 2 min and autoclaved at 121.1°C for 15 min. After cooling on ice, blended oyster tissue was blended again for 2 min to break down heat coagulated particles to a fine, creamy homogenate. Each strain of spore suspension was mixed into oyster homogenate with an inoculum ratio of 1 to 10 to give a final concentration of 10^4 to 10^5 spores per ml determined by MPN method. The mixture was homogenized for one additional minute so that the spore suspension was uniformly incorporated prior to thermal resistance experiments.

Procedure of thermal death time experiments

Thermal death time (TDT) experiments were carried out in 10 x 75-mm heat-resistant glass TDT tubes by the method of Lynt et al. (*22*). Each of the TDT tubes was filled with 1.1 ml of oyster homogenate using a 10-ml sterile pipet. The tubes were immediately flame sealed and stored in an ice bath. For each test temperature, 60 replicate TDT tubes were divided into six groups each with 10 tubes bound together and dropped into the bath simultaneously with a sinker attached to each group of tubes assuring complete immersion in the bath. Each group of 10 tubes was removed from the bath at 0.1- to 20-min intervals depending upon the test temperature and cooled in an ice bath. Water bath temperature was precalibrated in triplicate against a NBS thermometer to within 0.10°C and was monitored with a copper-constantan thermocouple. After cooling, TDT tubes were aseptically opened; the content of each tube was cultured in a separate tube of 8 ml TPGYT broth (TPGY plus 0.1% trypsin) containing a Durham tube. All cultures were incubated at 28°C and checked for growth every 2 or 3 d for 4 weeks until evidence of growth was observed. Tubes showing growth were streaked on TPGY agar plates and incubated in a BBL gaspak jar at 28°C for 4 d for confirmation of *C. botulinum* by morphological and biochemical tests. Both positive and negative growth tubes were tested for *C. botulinum* toxin (*14*). Tubes showing no signs of growth were sealed with a layer of vaspar and incubated for an additional 4 months. All tubes with no visible sign of growth at the end of the 4-month incubation period were considered sterile. Three replicate experiments were performed for each test temperature and the heating times required for the last positive culture or first all negative culture at each test temperature were averaged to construct TDT curves.

Toxin test

C. botulinum toxin was tested essentially as described by the Food and Drug Administration (*14*). Samples were diluted 1:1, 1:2, or 1:4 depending on sample concentrations. All samples were centrifuged to remove cells and solids. The supernatant was then filter sterilized and 0.5-ml aliquots were injected intraperitoneally into white mice (about 20 g) in duplicate. The mice were observed periodically for up to 72 h for symptoms of botulism.

Calculation

Corrections for thermal lag and lethality during lag were computed by the graphic method (*1*). Cooling lag time was similarly measured. The average of at least five heat penetration curves was plotted on inverted 3-cycle semilogarithmic paper for each temperature determination. Measurements of the rate of heat penetration were constructed by charting readings from a copper-constantan thermocouple located in the center of the 1.1 ml oyster homogenate in each TDT tube.

Corrected times for the longest heating period at which growth still occurred and the shortest heating period at which no growth occurred for all cultures at each process temperature were averaged. Average values were plotted on semilogarithmic paper with time on the logarithmic axis and temperature on the arithmetic axis. The TDT curve was then constructed so that all points representing the heating time for the last positive culture were below it, and all the points representing the first negative culture at each temperature were above it. Decimal reduction times (D) were calculated from the corrected data by the probability method of Schmidt (*27*):D = L.D. 50/ (log A - log 0.69). D is time in minutes at a given temperature to reduce the spore count by 1 log. L.D. 50 represents the time at which 50% of tubes were sterile. A is the initial number of organisms per tube, and 0.69 is a constant corresponding to the L.D. 50 point. The temperature necessary to bring about a 10-fold change in TDT or D value, i.e., the z value, was determined from the absolute value of the inverse slope of both TDT and heat resistance curves.

RESULTS AND DISCUSSION

Based on end-point destruction data, TDT curves for spores from strains of *C. botulinum* type E were constructed to best fit and are shown in Fig. 1 and 2. The Alaska and Minnesota strains of *C. botulinum* had relatively similar heat-resistance patterns, with the Minnesota more heat resistant (Fig. 1). The slope of the TDT curve of strain Alaska was slightly steeper than that of strain Minnesota. All three Chesapeake strains, G 21-5, 25V-1, and 25V-2, had similar heat-resistance curves; strain 25V-2 was slightly more heat sensitive (Fig. 1 and 2). All three strains had z values in the range of 5.3 to 6.7°C.

Fig. 3 shows corresponding D values from the 2-month incubation of five tested strains. The heat-resistance curve of strain Alaska was much steeper. D value calculations were based on subculture of TDT tubes for 2 months (Table 1). D values for these strains in oyster homogenate ranged from 2.00 to 8.96 min at 73.9°C, 0.73 to 2.69 min at 76.7°C, 0.25 to 1.03 min at 79.4°C, and 0.07 to 0.43 min at 82.2°C. Minnesota strain was the most heat resistant. Chesapeake Bay strains were relatively heat sensitive, particularly strain 25V-2. All of the tubes that received longest heating time in each treatment but were positive for growth also tested positive for the presence of toxin. All of the tubes that received the shortest heating time but were negative for growth also tested negative for the presence of toxin. The effect of delayed germination on calculated D values was studied by monitoring growth weekly for 2 months. All 2-week D values were constant through 4 months incubation except for strain G21-5 heated at 79.4°C (175°F) where D-value increased from 0.27 min at 2 weeks to 0.29 min when subcultured for 2 months. This indicates that subculture for 2 weeks was sufficient for determining D value except for strain G21-5 at a process temperature of 82.2°C.

TABLE 1. *Decimal reduction time (D) of* C. botulinum *type E spores in oyster homogenates.*

Temperature		Strain				
°C	(°F)	Alaska	Minnesota	G21-5	25V-1	25V-2
73.9	(165)	7.56	8.96	5.23	7.13	2.00
75.0[a]	(167)	3.85	5.28	2.38	3.40	1.28
76.7	(170)	1.53	2.69	1.01	1.42	0.73
79.4	(175)	0.41	1.03	0.29	0.58	0.25
82.2	(180)	0.07	0.43	0.11	0.20	0.08

[a] $D_{75°C}$ values were derived from Fig. 3.

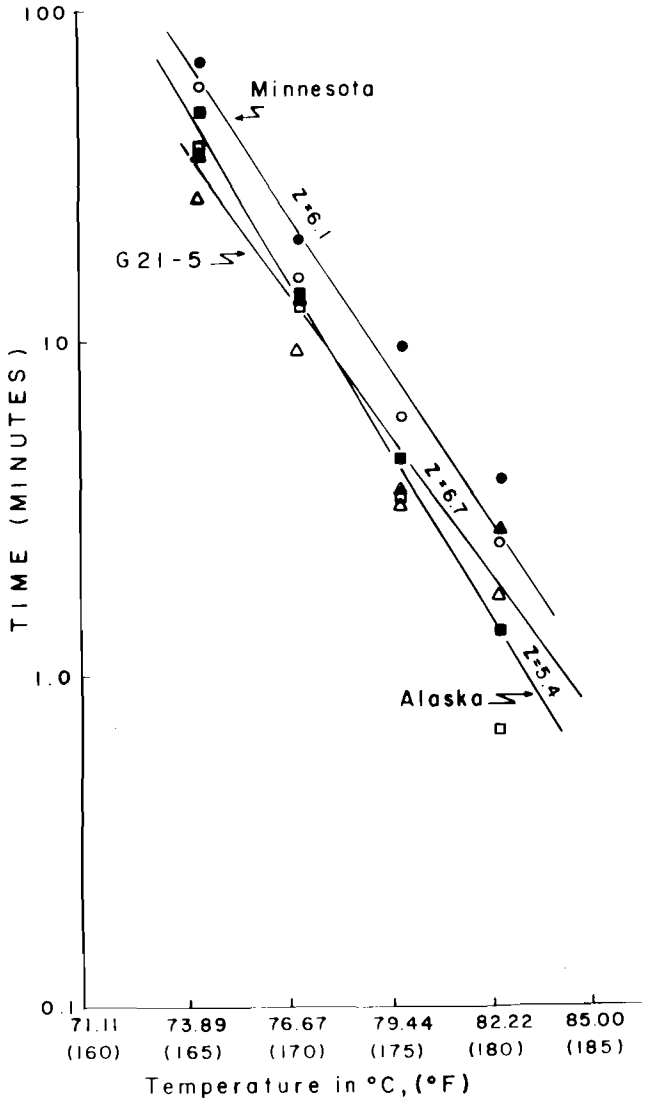

Figure 1. *Thermal death time curve for spores of* C. botulinum *type E (strain Minnesota, Alaska and G21-5). Opened circles, squares, or triangles represent the last positive cultures; closed circles, squares, or triangles the first negatives.*

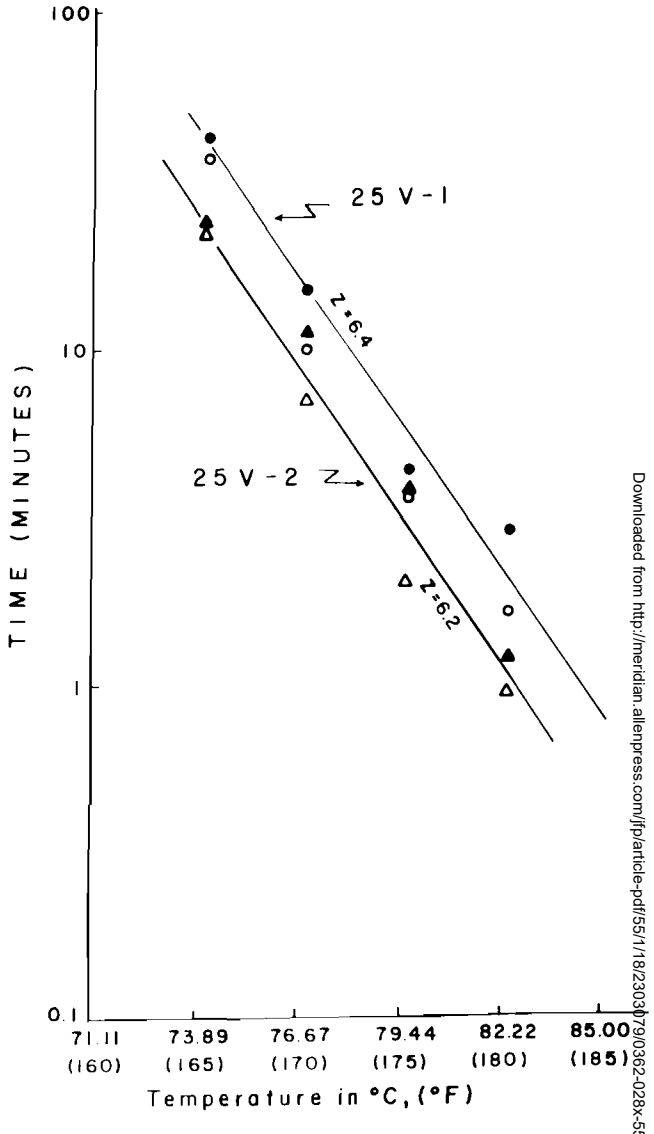

Figure 2. *Thermal death time curve for spores of* C. botulinum *type E (strain 25V-1 or 25V-2). Opened circles or triangles represent the last positive cultures; closed circles or triangles the first negatives.*

The z values calculated from TDT curves and heat resistance curves are summarized in Table 2. For strain Alaska, the z value was 5.4°C from the TDT curve and 4.2 from the heat resistance curve, whereas Minnesota and 25V-2 strains showed only minor D value differences between these two methods. Strain G21-5 demonstrated a great difference between z values determined by the two methods (6.7°C for the TDT curve and 5.3°C for the heat resistance curve). z values were lower when they were determined from heat resistance curves as compared to TDT curves. Similar results were reported by Lynt et al. (*22*) who obtained higher z values from TDT curves as compared with heat-resistance curves for *C. botulinum* type E strains in crab meat. The discrepancy might be due to the fact that TDT curves were constructed based on relatively low inoculum levels of heat-resistant spores, whereas heat resistance curves were derived from the large fraction of the population which could be relatively heat sensitive (*21*). As discussed by some researchers, several possible rationales might explain the difference in z values (*21,25*). These include clumping of cells, protection due to the presence of unhomogenized food ingredients, adherence of some spores to the TDT tube wall to protect them from "moist" heat, and differences in physiological condition among the spore population. Such factors would also affect D values.

C. botulinum type E could not multiply in oyster homogenate at refrigeration temperature below 3°C (data not shown). Heated *C. botulinum* type E spores did not grow and produce toxin at 7.8°C or below (*8,29*). However, refrigeration temperature did not reduce spore viability in oyster homogenate, and all spores survived after 4 months storage at 3.3°C. When the incubation temperature was increased to 28°C, *C. botulinum* type E strains (Alaska and 25V-1) demonstrated a 2- to 3-log cycle growth within 3 d in oyster homogenate with an initial inoculation of 10^2 to 10^4 spores per ml (Table 3).

The heat resistance of *C. botulinum* type E in oyster homogenate shown in this study was relatively lower than

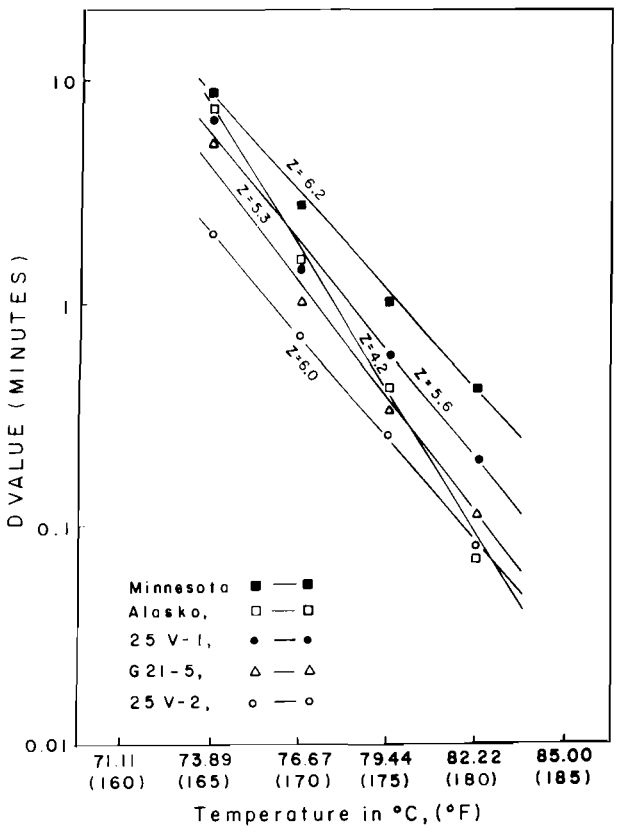

Figure 3. *Heat resistance curves for spores of five strains of* C. botulinum *type E.*

TABLE 2. *z Values in °C (°F) of* C. botulinum *type E spores obtained from different methods.*

Method	Strain				
	Alaska	Minnesota	G21-5	25V-1	25V-2
TDT curve					
Best-fiting	5.4(9.8)	6.1(11.0)	6.7(12.1)	6.4(11.5)	6.2(11.2)
Calculation	5.1(9.2)	6.6(11.9)	7.1(12.8)	6.7(12.0)	6.2(11.1)
Heat-resistance curve					
Best-fitting	4.2(7.6)	6.2(11.2)	5.3(9.6)	5.6(10.1)	6.0(10.8)
Calculation	4.2(7.5)	6.3(11.4)	5.5(9.9)	5.5(9.9)	5.9(10.7)

TABLE 3. *Growth of* C. botulinum *type E spores in oyster homogenate stored at 28°C.*

Strain	Count per ml during storage	
	0 d	3 d
25V-1	3.0×10^2	4.0×10^5
Alaska	2.0×10^4	2.8×10^6

that reported in the literature for other seafoods. In oysters $D_{79.4°C}$ for strains Alaska and G 21-5 were 0.41 and 0.29 min, respectively, as compared with 1.35 and 1.10, respectively, in crab meat (*22*). A $D_{79.4°C}$ for strain Alaska was found to be 5.08 in whitefish chubs (*10*). Low heat resistance in oyster homogenate is possibly due to a lower pH in oyster tissue.

Oyster composition such as moisture, lipid, salt, other minerals, and pH could cause fluctuation in the heat resistance of type E *C. botulinum* in oysters. In particular, low pH (6.1-6.2) could have a major effect in lowering heat resistance. Oysters harvested from different seasons or locations can vary in composition, such as glycogen, salt, and taurine. Other parameters such as freshness, method of oyster processing, and oyster handling after harvest all contribute to the composition and hence may affect the heat resistance of test organisms.

Based on heat-resistance data from this study, the safety of oyster pasteurization is a major concern. As we reported previously (*6*), oysters pasteurized at 72°C for 8 min or at 75°C for 8 min (unpublished data) retained the flavor and texture of freshly shucked oysters and the shelf life was extended up to 3 months when stored at 0.5°C. Results from this study show pasteurization under conditions used here would provide a 2D process for strains Alaska and 25V-1, a 3D process for G21-5, a 6D process for 25V-2, and only a 1.5 D process for strain Minnesota. A recent report from Bucknavage et al. (*3*) indicated that the most heat-resistant Beluga strain among five strains they tested had a $D_{80°C}$ of 0.78 in oysters, which would be equal to $D_{75°C}$ of 4.98. This is slightly more heat sensitive than strain Minnesota in this study. The heat resistance of *C. botulinum* type E obtained in this study was based on the inoculation of this organism into autoclaved oyster homogenate without any other additives. The chlorination of oysters at 50 ppm chlorine prior to pasteurization and storage at 0.5°C could reduce the incidence of *C. botulinum* type E present in pasteurized oysters (*24*). In addition, the heat resistance during pasteurization of this organism in prechlorinated, shucked oysters might be much lower than that in autoclaved oyster homogenate because of lower pH and chlorination injury. *C. botulinum* type E was found to be inhibited at pH 5.7 and 8°C or at pH 6.2-6.4 and 5°C (*13,28*). In our oyster pasteurization process (*6,24*), the combination of chlorination, low pH, and refrigeration temperature could render the thermal resistance of *C. botulinum* type E lower than that found in this study (*6,24*). Additional studies of oyster pasteurization processes using inoculated packs are warranted. The assurance of safety from type E *C. botulinum* is a key factor in determination of a successful oyster pasteurization process.

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